

Optimization of Carbon and Nitrogen Sources for Extracellular Polymeric Substances Production by *Chryseobacterium indologenes* MUT.2

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Background: Bacterial Extracellular Polymeric Substances (EPS) are environmental friendly and versatile polymeric materials that are used in a wide range of industries such as: food, textile, cosmetics, and pharmaceuticals. To make the production process of the EPS cost-effective, improvements in the production yield is required which could be implemented through application of processes such as optimized culture conditions, and development of the strains with higher yield (e.g. through genetic manipulation), or using low-cost substrates.

Objectives: In this work, the effects of carbon and nitrogen sources were studied in order to improve the EPS production by the submerged cultivation of *Chryseobacterium indologenes* MUT.2.

Materials and Methods: The mesophilic microorganism *Chryseobacterium indologenes* MUT.2, was grown and maintained in the Luria Bertani agar. The initial basal medium contained: glucose (20 g.L⁻¹), yeast extracts (5 g.L⁻¹), K₂HPO₄ (6 g.L⁻¹), NaH₂PO₄ (7 g.L⁻¹), NH₄CL (0.7 g.L⁻¹), and MgSO₄ (0.5 g.L⁻¹). For evaluating the carbon and nitrogen sources' effect on the fermentation performance, cultures were prepared in 500 mL flasks filled with 300 mL of the medium. The single-factor experiments based on statistics was employed to evaluate and optimize the carbon and nitrogen sources for EPS production in the liquid culture medium of *Chryseobacterium indologenes* MUT.2.

Results: The preferred carbon-sources, sucrose and glucose, commonly gave the highest EPS production of 8.32 and 6.37 g.L⁻¹, respectively, and the maximum EPS production of 8.87 g.L⁻¹ was achieved when glutamic acid (5 g.L⁻¹) was employed as the nitrogen source.

Conclusions: In this work, the culture medium for production of EPS by *Chryseobacterium indologenes* MUT.2 was optimized. Compared to the basal culture medium in shake-flasks and stirred tank bioreactor, the use of optimized culture medium has resulted in a 53% and 73% increase in the EPS production, respectively.

Keywords: Carbon source; *Chryseobacterium indologenes*; Extracellular polymeric substance; Medium composition; Nitrogen source; Stirred tank bioreactor

1. Background

Bacterial Extracellular Polymeric Substance (EPS) are environmental friendly polymeric materials that are used in a wide range of industrial applications (food, textile, cosmetics, pharmaceuticals, etc.) (1). Traditional EPS derived from other natural sources (i.e. plants, algae, and animals) fail to perform in some applications, in contrast, bacterial EPS may demonstrate new and improved properties. Furthermore, compared to synthesis of EPS by higher plants and algae, their microbial production is more productive and less resource-intensive. Moreover, microbial pro-

duction enables the control of process conditions to obtain higher yields and desired properties (2). However, due to their high production costs, only a few bacterial EPS are commercially available (e.g. xanthan, gellan gum, hyaluronic acid). To make the process more cost-effective, improvements in the product yield is necessary, which, could be achieved through optimization of the culture condition, development of the high yield strains (e.g. through genetic manipulation), or application of the low-cost substrates (3). Nutritional and environmental conditions strongly affect microbial EPS' synthesis and composi-

tion (4). Carbon and nitrogen sources generally play a significant role, because these nutrients are directly linked to the cell proliferation and metabolite biosynthesis (5, 6). Also, the nature and concentration of the carbon source can regulate the secondary metabolism through phenomena such as catabolic repression (7).

A number of statistical experimental design combined with the response surface methodology (RSM), such as factorial design, uniform design, Central Composite Design (CCD), and Box-Behnken Design (BBD) were applied for optimization of the fermentation conditions (1). M. Khani *et al.* (1) have used CCD for optimization of the operating parameters for EPS production by *C. indologenes* MUT.2. But, as far as we know, there is limited knowledge regarding nutritional requirement for EPS production by *C. indologenes* MUT.2. In addition, there hasn't been any report with respect to the medium optimization improvement for EPS production. *C. indologenes* MUT.2 was isolated from garden soil and hot spring water samples were collected from Arak city in Iran in order to investigate the corrosion inhibition by EPS. The EPS produced by the isolated *C. indologenes* MUT.2 was found to have high anti-corrosive properties (8). The electrochemical behavior of this new type of anti-corrosive EPS has been examined using electrochemical techniques and surface analytical examinations (FT-IR, EIS) (8). Both carbon and nitrogen sources were optimized in the present study by employing statistical method based on the single-factor experiments in order to improve the EPS production by *C. indologenes* MUT.2.

2. Objectives

In this study, for the first time, the effects of carbon and nitrogen sources were studied in order to improve the EPS production by submerged cultivation of *C. indologenes* MUT.2. The obtained information is considered to be fundamental and useful for development of the *C. indologenes* MUT.2 cultivation process, as well as an efficient production of the EPS on a laboratory scale stirred tank bioreactor.

3. Materials and Methods

3.1. Microorganism and Inoculum Preparation

The mesophile microorganism *C. indologenes* MUT.2 was kindly provided by the Biological Science and Technology Department, Malek Ashtar University, Tehran, Iran. The strain was maintained on nutrient agar at 4°C and sub-cultured monthly. The strain was grown and maintained in LB-agar with the following

composition: 5.0 g.L⁻¹ sodium chloride, 5.0 g.L⁻¹ yeast extract, 10.0 g.L⁻¹ tryptone, and 15.0 g.L⁻¹ agar. During the preparation, the pH of the media was adjusted at 7.0 and then sterilized (121°C/15min). Following to the 24 h of growth at 30°C, the culture was maintained at 4°C (9).

Inoculum cultures were prepared in the LB-broth containing the following components as % W/W-final concentration: 0.5 sodium chloride, 1.0 tryptone, and 0.5 yeast extract (10). The pH of the medium was adjusted to the 7.0 before autoclaving (121°C/15 min). *C. indologenes* MUT.2 cells were inoculated in 250-mL Erlenmeyer flasks containing 150 mL of LB-broth at 30±2°C for 24 h. The flasks were placed in an orbital shaker (Tecnal model. TE-424, Tehran, Iran) at 170 rpm. Cell growth was monitored spectrophotometrically (PerkinElmer model Lambda 20) by measuring the optical density at 600 nm after 24 h of incubation until the cell count has reached 10⁷ CFU.mL⁻¹.

3.2. Culture Medium

The initial basal medium used contained glucose (20 g.L⁻¹), yeast extracts (5 g.L⁻¹), K₂HPO₄ (6 g.L⁻¹), NaH₂PO₄ (7 g.L⁻¹), NH₄CL (0.7 g.L⁻¹), and MgSO₄ (0.5 g.L⁻¹). The medium constituents and glucose were sterilized separately by autoclaving at 121°C for 15 min, and were mixed thoroughly before inoculation. The chemicals used in this study were of reagent grade.

3.3. Carbon and Nitrogen Source Screening

For evaluating the effect of carbon and nitrogen sources on fermentation performance, cultures were conducted in 500 mL shake flasks filled with 300 mL of the medium. Then, the bacterial pre-culture medium (6% , V/V) was inoculated in the flasks and incubated for 96 h. The flasks were held at 30°C on a rotary shaker at 170 rpm.

3.4. Fermentation Conditions

The fermentation medium was inoculated with 6% (V/V) of the seed culture and then cultivated at 30°C in a 3 liter stirring tank bioreactor (Bioflow III, New Brunswick Scientific Co., New Brunswick, NJ, USA). Unless otherwise specified, fermentation were conducted under the following condition: temperature at 30°C, aeration rate 1.0 vvm, agitation speed 250/min, the initial pH 7.0, and the working volume 2 l.

3.5. Analysis of Biomass

The bacterial dry cell biomass was collected by

centrifuging 1 mL culture samples at 11000 g for 10 min (Sigma 1-16K, No.12134). The centrifuged cell pellet was washed twice with distilled water and dried at 80°C in an oven for 24 h to obtain cell dry weight (CDW). CDW was calculated using a gravimetric method and was expressed as g.L⁻¹.

3.6. Analysis of EPS

Mycelial pellets were separated by centrifugation (Sigma 6-16K, No.12256) at 16000 g for 10 min and the supernatant was mixed with three volumes of the chilled absolute ethanol to precipitate the EPS. The sample solutions were stored at 4°C for 24 h to enhance the precipitation. Finally, the EPS were recovered by centrifugation at 9000 g for 15 min and dried at room temperature. EPS production was calculated using a gravimetric method and was expressed as g.L⁻¹.

4. Results

4.1. Effect of Carbon Sources

The following set of experiments were done in order to investigate the effects of the various types of carbon sources on the cell growth of *C. indologenes* MUT.2 and production of EPS. In all cases, nitrogen was provided as yeast extract at the same concentration that was used in the basal medium, where each carbon source was added to the basal medium at 20 g.L⁻¹ instead of glucose. (Figure 1) shows the time profile of the biomass concentration (A) and EPS production (B) for the cultures carried out with all different types of the tested carbon sources as well as the culture without addition of the any type of carbon source as the control. The results obtained from shake-flask experiments revealed that, the change in the employed

Table 1. The effect of the different carbon sources on *C. indologenes* MUT.2 growth and extracellular polymeric substance (EPS) production during cultivation in the shake-flasks for 96 h at 30°C. The highest cell mass, EPS and other related factors were observed when sucrose was used a carbon source

Carbon sources	Dry cell weight (g.L ⁻¹)	EPS (g.L ⁻¹)	EPS/CDW	Final pH
Control*	0.41	0.22	0.53	7.28
Xylose	1.32	5.63	4.26	6.53
Galactose	1.40	3.58	2.55	6.64
Glycerol	1.48	6.08	4.10	6.32
Glucose	1.52	6.37	4.19	6.15
Fructose	1.29	4.96	3.84	6.81
Maltose	1.56	2.37	1.50	6.94
Sucrose	1.85	8.32	4.49	5.98
Lactose	1.37	4.12	3.00	6.56
Mannose	1.05	2.51	2.39	7.17
Starch	0.98	2.07	2.11	7.35
Dextrin	1.12	3.15	2.81	7.22

*Control culture without any carbon source

carbon source has affected both the amount of the produced biomass and the EPS production. Although galactose, maltose, and lactose gave good mycelial growth, they led to low EPS yields. The preferred carbon sources; sucrose and glucose, generally resulted in the highest EPS production of 8.32 and 6.37 g.L⁻¹, respectively as shown in (Table 1).

4.2. Effect of the Nitrogen Source

To investigate the effect of the nitrogen source on EPS production and mycelial growth, eight different nitrogen sources (N source) were examined (Figure 2). These N-sources were added on the basis of an equivalent N-content. Although yeast extract and glycine

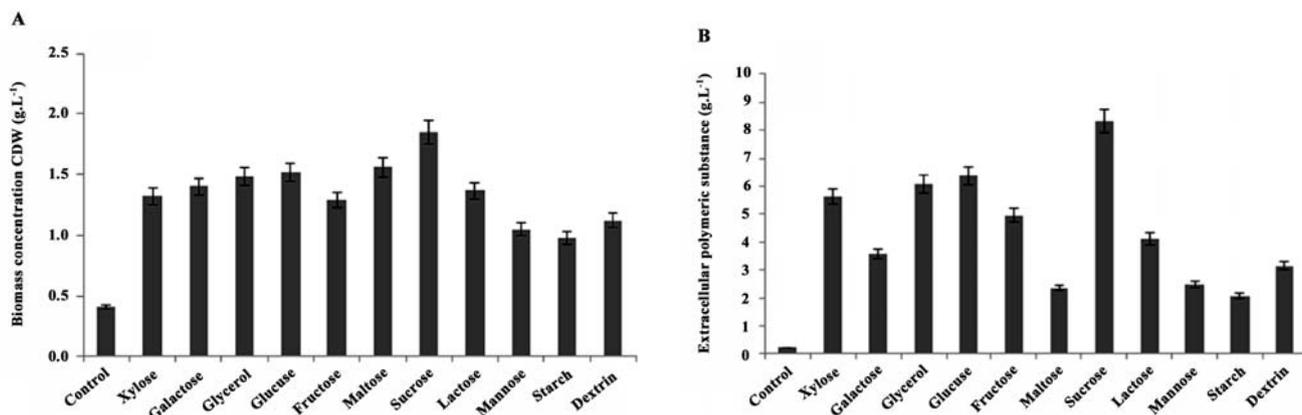


Figure 1. A: The effect of different carbon sources on the cell growth of *C. indologenes* MUT.2 and B: EPS production after 96 h of cultivation. sucrose was found to have the best effect on EPS production

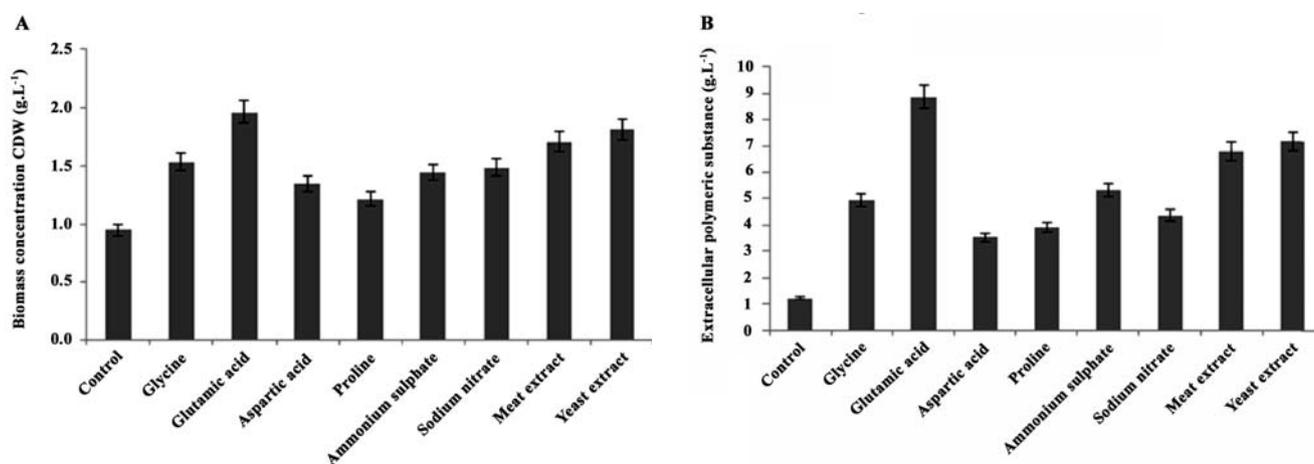


Figure 2. A: Effect of the different nitrogen sources on the *C. indologenes* MUT.2 cell growth and B: EPS production following 96 h of cultivation. Glutamic acid was found to have the best effect on EPS production

were favorable for the mycelial growth of the *C. indologenes*, the maximum EPS production of 8.87 g.L⁻¹ was achieved when glutamic acid (5 g.L⁻¹) was employed (Figure 2B). Improvement in the cell growth, EPS production, and EPS/CDW ratio through application of the different nitrogen sources are summarized in (Table 2).

4.3. Cultivation of *C. indologenes* MUT.2 in Shake-Flasks with the Optimized Medium

Using all optimized medium components, trials in a series of the shake-flasks was done using 20 g.L⁻¹ sucrose as the carbon source and 5 g.L⁻¹ glutamic acid as nitrogen source. The time course of the cell growth,

EPS production, and pH values are shown in (Figure 3). The maximal EPS production was achieved as: 8.9 g.L⁻¹ after 3.5 days of the fermentation, while, the maximum mycelial yield was about 1.96 g.L⁻¹ achievable after 3.5 days. This indicates a 53% increase in the EPS production using the optimized medium (8.9 g.L⁻¹) compared to EPS production using the basal medium (5.8 g.L⁻¹), as previously described. In addition, similar increase in the biomass concentration was achieved using the optimized medium.

4.4. Fermentation in the Stirring Tank Bioreactor

Although shake flasks are the most frequently used bioreactors in biotechnology for an initial process development, very little is known regarding their characteristics from an engineering point of view (11, 12). There are several reports in the literature regarding the scaling-up of processes from the shake flasks to the

Table 2. The effect of the different nitrogen sources on the *C. indologenes* MUT.2 growth and its extracellular polymeric substance (EPS) production during cultivation in shake-flasks for 96 hours at 30°C. The highest cell mass, EPS and other related factors were observed when glutamic acid was used as nitrogen source

Nitrogen sources	Dry cell weight (g.L ⁻¹)	EPS (g.L ⁻¹)	EPS/CDW	Final pH
Control*	0.95	1.21	1.27	6.07
Glycine	1.53	4.95	3.23	6.51
Glutamic acid	1.96	8.87	4.52	6.61
Aspartic acid	1.34	3.51	2.61	6.38
Proline	1.21	3.92	3.23	5.96
Ammonium sulphate	1.44	5.32	3.69	5.81
Sodium nitrate	1.48	4.35	2.93	5.49
Meat extract	1.70	6.78	3.98	6.32
Yeast extract	1.81	7.17	3.96	6.41

*Control culture without any nitrogen source

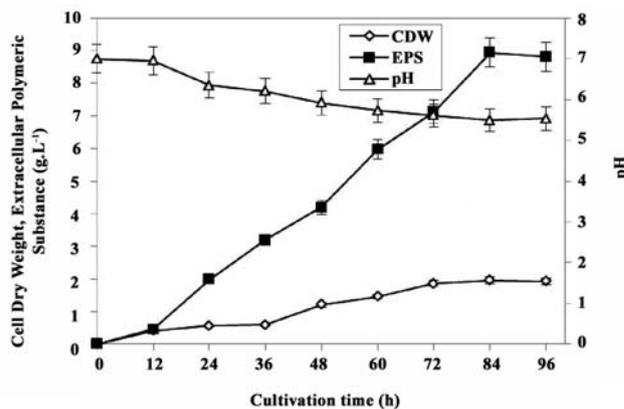


Figure 3. Batch cultivation of the *C. indologenes* MUT.2 in the shake flasks using the optimized medium

stirred tank bioreactors (13, 14). Therefore, the bioreactor fermentation process was developed on the basis of data obtained from shake flasks cultivation experiments. The production of EPS was studied in a 3 liter bioreactor using the basal and the optimized culture medium.

Figure 4 shows the time courses of mycelial growth and EPS production by *C. indologenes* MUT.2 in a 3 liter stirred tank fermenter with the basal medium and the optimized culture medium. In the basal medium, the EPS concentration reached a maximum level of 5.84 g.L⁻¹ after 3 days, while, maximum mycelial concentration was 1.59 g.L⁻¹ after 3 days (Figure 4A). In an optimized culture medium as shown in (Figure 4B), the mycelial growth was continuously increased towards the end of fermentation and its final mycelial concentration has indicated an amount of almost 1.98 g.L⁻¹ at day 3.5. The initial pH of the fermentation broth slowly decreased from 7.00 to 5.02. The EPS production reached 10.15 g.L⁻¹ after 3.5 days of the

fermentation, which is 1.7 times higher than that of fermentation in the basal medium. Optimization of the operating parameters (*e.g.* agitation, aeration, and dissolved oxygen tension) in the bioreactor fermentation deserves further investigation, which is being carried out in our laboratory.

5. Discussion

The results obtained in the present study are in agreement with the general concept expressed in the early studies (15, 16) which have reported the highest exopolysaccharides yield using fructose, sucrose, and glucose as carbon sources. Recently, Zhang *et al.* (17), studied the production of gellan exopolysaccharide by growing strains of *Sphingomonas paucimobilis* QHZJ UJW in the medium containing various carbon sources such as glucose, sucrose, maltose, malt extract powder, and vegetable oil. They found that the highest EPS production of the strain in the sucrose containing medium. Qiang *et al.* (18) have used RSM for EPS production by *Klebsiella* sp. H-207, isolated from activated sludge. A maximum EPS yield of about 15.05 g.L⁻¹ was achieved under optimized conditions in terms of both the medium composition and culture condition, which consisted of sucrose 31.93 g.L⁻¹, KNO₃ 2.17 g.L⁻¹ and K₂HPO₄ 5.47 g.L⁻¹, a seed age of 13 h, with an inoculum size of 10.6% and an incubation temperature of 28.9°C. Screening of the most significant fermentation parameters affecting levan production was done by the statistical designs for the microbial strain *Pseudomonas fluorescens* NCIM 2059. Six nutritional variables (*i.e.* sucrose, casein peptone, NH₄Cl, KH₂PO₄, MgSO₄, and NaNO₃) were studied to define an optimal medium. A significant increase in the levan yield from 5.27 up to 15.42 g.L⁻¹ under these conditions was found (19). In a recent study, a novel bacterium strain BM39, associated with *Pantoea* sp., was isolated from sediments of Tyrrhenian Sea. The strain was selected for its ability to produce a very high levels of the glucan EPS. Kinetic studies of EPS production by the strain BM39 cultivated in the shaken cultures using a medium containing 80 g.L⁻¹ of sucrose (EMS medium) and 80.0 g.L⁻¹ of fructose (EMF medium) as carbon sources, was shown an EPS production the amounts of which was estimated as 11.82±1.06 g.L⁻¹ and 11.05±1.17 g.L⁻¹, respectively, while the productivity was 0.39±0.04 g.L⁻¹.h and 0.37±0.04 g.L⁻¹.h⁻¹ (20). Bounaix *et al.* (21) have clearly demonstrated a high biodiversity of EPS produced by sourdough LAB strains with sucrose substrate (21).

In this work, culture medium for production of EPS by *C. indologenes* MUT.2 was optimized. Compared

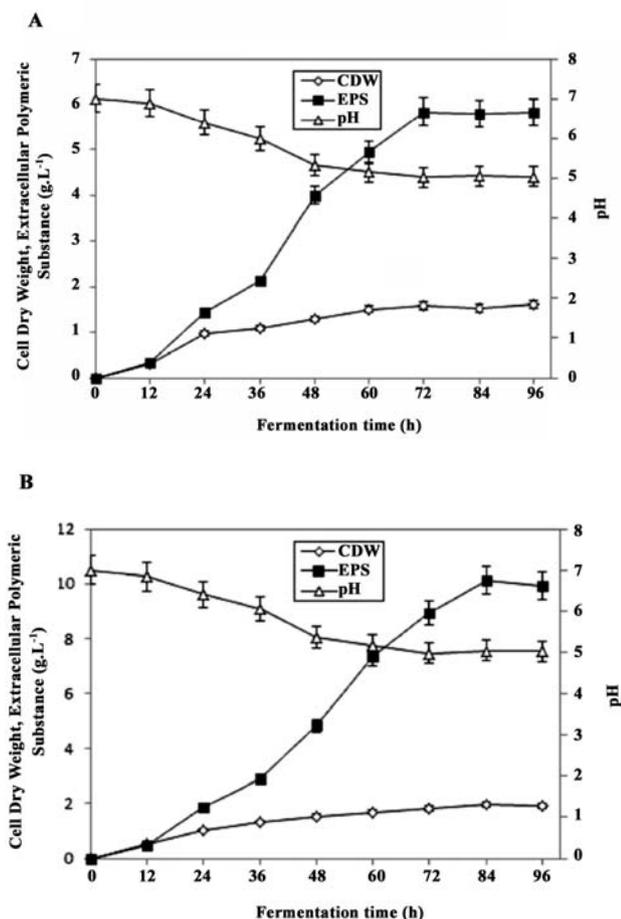


Figure 4. A: Batch fermentation of the *C. indologenes* MUT.2 in the stirred tank bioreactor in the presence of the basal medium and B: in the optimized medium

with the basal culture medium in shake-flasks and stirred tank bioreactor, the use of optimized culture medium has resulted in a 53% and 73% increase in EPS production, respectively. No reports are currently available in the literature regarding the optimization of the extracellular polymeric substance production by *C. indologenes* MUT.2 in the submerged culture. Our study provides an important information for the large-scale production of the bioactive natural products such as EPS by *C. indologenes* MUT.2, which can be used as an functional anti-corrosive.

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